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# Induction of cytokines, MMP9, TIMPs, RANKL and OPG during orthodontic tooth movement

Melissa Grant, Jay Wilson, Peter Rock and Iain Chapple

\*School of Dentistry, College of Medical and Dental Sciences, University of Birmingham, UK

Correspondence to: Melissa M. Grant, School of Dentistry, College of Medical and Dental Sciences, University of Birmingham, St Chads Queensway, Birmingham B4 6NN, UK.

E-mail: [m.m.grant@bham.ac.uk](mailto:m.m.grant@bham.ac.uk)

**SUMMARY** This controlled longitudinal intervention study investigated changes in cytokines and biomarkers of bone and tissue metabolism within gingival crevicular fluid (GCF) from patients undergoing orthodontic treatment. GCF was collected on Periopaper™ strips (Oraflow Inc., USA) from 20 volunteers at baseline, before tooth extraction and appliance placement and then at intervals during orthodontic treatment. Samples were taken 10 weeks following first appliance placement: at four hours; 7 days; and 42 days after application of distalising forces to maxillary canine teeth. Cytokines (GM-CSF, interferon-gamma, IL-1beta, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and TNFalpha), tissue biomarkers (MMP-9, TIMP-1 & 2) and bone metabolism indicators (RANKL and OPG) were measured in GCF using multiplex assays. Tension sites adjacent to canines showed significant increases in IL-1beta, IL-8, TNFalpha, MMP-9 and TIMPs 1 and 2 across all time points following force application, while compression sites exhibited increases in IL-1beta and IL-8 after 4 hours, MMP-9 after 7 and 42 days and RANKL after 42 days. These data demonstrate that high levels of pro-inflammatory cytokines and biomarkers of tissue and bone metabolism in GCF are associated with orthodontic force application. Elevated levels were evident at 4 hours but continued for periods of up to 6 weeks. The data suggest that GCF biomarker analysis may help optimise orthodontic forces for individual patients.

## Introduction

The application of orthodontic force to a tooth creates areas of tension and compression in the periodontal ligament (PDL) that affect remodelling of the ligament, alveolar bone and gingivae. Following mechanical stress, changes to the vascularity and blood flow within the PDL are induced by signalling molecules. The cascade commences with arachadonic acid metabolites (eicosanoids), neurotransmitters, (substance P and calcitonin gene-related peptide) and second messengers such as cyclic AMP, phosphoinositol phosphates and diacyl glycerol. These trigger the release of cytokines, growth factors and colony stimulating factors, which in turn affect bone and tissue modulating species such as the receptor for NF kappa B Ligand (RANKL), osteopontin (OPG), matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs).

Cytokines and eicosanoids are involved in all stages of inflammation, from initiation to resolution. Pro-inflammatory cytokines, such as interleukin (IL)-1beta, IL-2, IL-5, IL-6, IL-8, TNFalpha, interferon-gamma and GM-CSF, induce the classical hallmarks of inflammation through vasodilation and tissue invasion by leukocytes; whereas anti-inflammatory cytokines, such as IL-4 and IL-10, are involved in its resolution. IL-1beta is released mainly by activated monocytes, after which it

participates in the acute phase response to induce bone resorption (Jandinski *et al.*, 1988). Following application of elastic separators mesially to the first molars (Dudic *et al.*, 2006; Giannopoulou *et al.*, 2006) or distal movement of maxillary first premolars after removal of the second premolars (Ren *et al.*, 2007), IL-1beta increased at sites of tension and compression. Giannopoulou *et al.*, (2006) demonstrated that this increase was mirrored by increases in substance P and pain intensity. Distal movement of canines by various forces (Yamaguchi *et al.*, 2006; Iwasaki *et al.*, 2001) also produced increases in IL-1beta levels within 1 day. Iwasaki *et al.*, (2001) reported that IL-1beta levels correlated with speed of tooth movement. IL-8, IL-6 and TNFalpha have also been studied as they also affect osteoclastogenesis. IL-8 increased after 4 days (Maeda *et al.*, 2007CIT0025) but decreased to below-baseline levels by 7 days (Basaran *et al.*, 2006). Other studies have shown that TNF alpha and IL-6 are increased within 24 hours of force application (Ren *et al.*, 2002 & 2007; Uematsu *et al.*, 1996), perhaps indicating different roles for IL-8 and IL-1beta, IL-6 and TNF alpha (Ren and Vissink, 2008).

IL-1, TNF-alpha and IL-6 stimulate bone resorption through increased regulation of RANKL. RANKL is produced by osteoblasts and binds to RANK (receptor activator of nuclear factor kappa-B) on osteoclast precursors causing maturation in osteoclasts. However, this is offset

by osteoblastic production of OPG, which serves as a decoy receptor for RANKL, thereby inhibiting osteoclast differentiation (Boyle *et al.*, 2003). Kawasaki *et al.*, (2006) and Nishijima *et al.*, (2006) investigated the levels of RANKL and OPG in GCF from patients undergoing orthodontic treatment to distalise upper canines. Samples were obtained 1 hour, 1 day and 7 days following force application. Both studies showed that RANKL was increased and OPG decreased after 1 day at sites of compression according to enzyme-linked immunosorbent assays (ELISAs). Garlet *et al.*, (2007) used real-time PCR technology to study RANKL and OPG expression in the PDL of extracted teeth that had been exposed to forces of 7N for 7 days using Hyrax rapid expansion appliances. Greater levels of RANKL transcripts were found at sites of compression than tension and greater levels of OPG transcripts at sites of tension than compression. Both RANKL and OPG were increased at sites of tension and compression in comparison to control teeth.

Soft tissue remodelling has been investigated following orthodontic tooth movement. The soft tissues are metabolised by various enzymes, including matrix metalloproteinases (MMPs) and the tissue inhibitors of matrix metalloproteinases (TIMPs). Collagenases, MMP-1 and MMP-8, degrade collagen fibres, whereas gelatinases, such as MMP-2 and MMP-9, degrade denatured collagen, complementing the collagenases. In humans, GCF MMP-1 and MMP-8 (Cantarella *et al.*, 2006; Ingman *et al.*, 2005; Apajalahti *et al.*, 2003), MMP-2 and MMP-9 (Cantarella *et al.*, 2006; Takahashi *et al.*, 2006) and TIMP-1 (Takahashi *et al.*, 2006; Bildt *et al.*, 2009) have all been shown to increase at sites of compression and tension.

Burstone (1962), proposed three phases of tooth movement: phase 1, from 24 hours to 2 days following force application represents rapid movement of the tooth within its bony socket; during phase 2, from 20 to 30 days, there is no movement, as the socket is remodelled by removal of compressed tissue that may be necrotic or hyalinized. In phase 3 there is acceleration of tooth movement, with sites of tension showing bone deposition and compressed sites removal of necrotic material. Hyalinisation in the second phase is proportional to the applied force and thus optimal orthodontic forces are recommended that cause maximal tooth movement with minimal tissue damage.

In the present study, we used 100g to move maxillary canines and calculated the rate of tooth movement. GCF samples were used to measure cytokines and biomarkers of tissue and bone metabolism. Second molars were used as controls as they were not subjected to direct force, being the teeth furthest from the site of force application. Biomarkers were measured using the multiplex Luminex platform, enabling multiple biomarkers to be measured on the same samples. By using this method, simultaneous quantitative

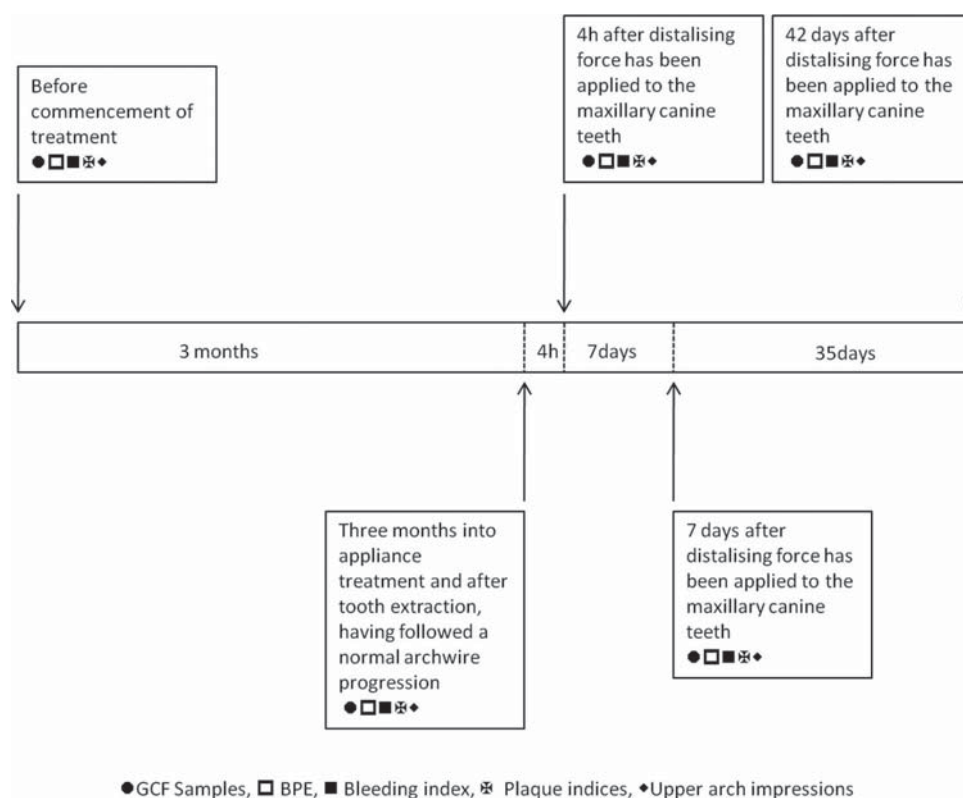
detection of multiple protein species is achieved (for a review of this technique please see: Vignali 2000). We aimed to map changes in GCF composition for three different groups of mediators simultaneously to temporal changes in tooth movement at sites of tension and compression in the same patients, with a view to enhancing understanding of the dynamic physiological changes induced by the application of orthodontic force.

## Material and methods

Twenty-one consecutive patients aged between 12 and 20 years with good systemic health were invited to take part in the study when they attended Birmingham Dental Hospital to commence orthodontic treatment. Ethical approval was obtained from the Coventry Research Ethics Committee (LREC 08/H1210/72). Inclusion criteria were the removal of both upper first premolar teeth and possessing fully erupted upper canines. Patients were excluded if they were previous smokers, had full mouth plaque and bleeding scores >20%; probing depths >4mm; radiographic evidence of periodontal bone loss; or had taken a course of anti-inflammatory or antimicrobial drugs within 1 month.

Informed consent was obtained at the first study appointment and patients underwent a detailed periodontal examination to record a plaque index—Turesky modification of the Quigley Hein index (Lobene *et al.*, 1982) and bleeding score (1 for bleeding and 0 for no bleeding). They also had GCF samples taken from the test teeth (the maxillary canines) prior to the examination of periodontal tissues. Upper arch alginate impressions were then taken to complete the baseline assessment.

During the next few weeks the maxillary first premolar teeth were extracted and one week was allowed for healing, prior to fitting a straightwire appliance. MBT prescription brackets, (3M Unitek, UK) and elastomeric modules (TP orthodontics, UK) were used, along with the archwire sequence 0.014 Nickel Titanium→0.018 Nickel Titanium→0.018 Stainless Steel. Archwire changes were made 6 and 10 weeks following appliance placement; the 0.018 Stainless Steel archwire was placed at 10 weeks. At the 10-week visit the baseline examination, involving GCF sampling, plaque index and bleeding score was repeated and upper arch impressions were taken, prior to application of a distalizing force to the canine teeth. Following sample collection, a 9mm nickel-titanium closing coil spring was placed between hooks on the brackets of the upper canines and the first molars with the aid of a ligature, which was tightened until the force produced was 100g (measured with a Correx tension gauge, Correx, Switzerland). GCF collection, detailed periodontal examinations, upper arch impressions and force adjustments were done in sequence after 4 hours, 7 days and 42 days (Figure 1).



**Figure 1** Schematic representation of study.

Canine movement was measured on the models using digital calipers twice, 3 months apart, to ensure intra-examiner consistency (% coefficient of variation = 0.17%), and the mean value used.

GCF volume was measured using a pre-calibrated Periotron 8000 (Chapple *et al.*, 1999 a and 1999b). Periotron strips were placed into the gingival crevice at the tension and compression aspects of test and control teeth until gentle resistance was felt. Strips were left in-situ for 30 seconds and removed prior to immediate placement in the Periotron 8000 for volume measurement. Samples were then combined for compression (left and right distopalatal sites) and tension (left and right mesiobuccal sites) for both canines and second molars. Each pair of strips was eluted into sterile PBS (200 µl) and stored under liquid nitrogen until assay.

GCF samples were thawed and biomarkers were detected using Luminex multi-analyte technology. Samples were incubated with antibodies immobilized on colour-coded microparticles, to detect the molecules of interest, washed to remove unbound material and then incubated with biotinylated antibodies to the molecules of interest. After further washing a streptavidin-phycoerythrin conjugate that binds to the biotinylated antibodies was added before a final washing step. The Luminex analyzer determined the magnitude of phycoerythrin derived signal in a microparticle specific manner (Table 1).

Differences in individual GCF analytes across the course of the study were analysed by paired non-parametric statistics (Kruskal–Wallis). Correlations were analysed by Spearman Rank Sum analysis. Graphpad Prism version 3.03 was used for all statistical analyses.

## Results

Volunteers were 20 consecutive patients attending the Birmingham Dental Hospital to begin a course of orthodontic treatment. During the 6-week observation period

**Table 1** Volume of GCF used per analyte and identification of manufacturer of each analyte kit. R&D systems (Abingdon, UK), Millipore (Watford, UK) and Invitrogen (Paisley, UK).

Analytes	Volume of GCF extract	Manufacturer	Limit of detection
TIMPs (1 & 2)	1µl	R&D	1: 0.6pg/ml; 2: 4.6 pg/ml
MMP-9	1µl	R&D	11pg/ml
OPG	25µl	Millipore	2.3pg/ml
RANKL	25µl	Millipore	5pg/ml
Cytokines (GM-CSF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN α, TNFα)	50µl	Invitrogen (Ultrasensitive kit required)	All <1pg/ml

the canine teeth moved a total of  $2.12 \pm 0.28$  mm (mean  $\pm$  SEM) towards the second premolars (Figure 2A). There were differences in the speed of movement at different times during treatment, as shown by the gradient of the graph between time points (Figure 2B); the greatest velocity being during the four hours following force application.

Clinical measurements were taken at each time point (Table 2 and Supplemental Figure 1) GCF volume was significantly increased over baseline at both tension and compression sites. There were no significant changes in GCF volume at control teeth sites at any time point. Variations in plaque index were not significant at tension or compression sites of canines or second molars at any time point. The bleeding index was found to be significantly elevated only at canine tension sites when arch-wires had been placed but before force was applied, reflecting the trend in plaque index changes in this period. There was a significant weakly

positive correlation between GCF volume and plaque index at sites of tension ( $r=0.6$   $P=0.004$ ) and compression ( $r=0.48$   $P=0.03$ ) for the canines when arch-wires were placed but before distalizing force had been applied. GCF volumes did not change significantly at the canines compared to the second molars; nor was there significant correlation between the speed of movement and either GCF volume, plaque or bleeding indices.

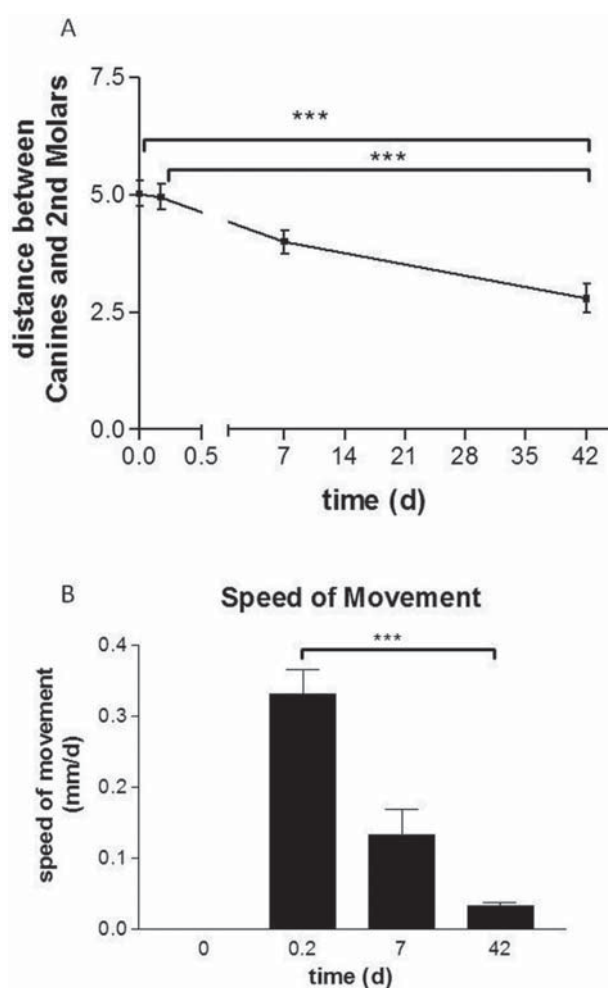
Temporal changes in GCF levels of ten cytokines were measured as 'amount per 30-second sample time' (Chapple *et al.*, 1999b) for samples collected around teeth undergoing orthodontic tooth movement. Four cytokines (IL-2, IL-4, IL-5 and IL-10) were below the limits of detection (Capelli *et al.*, 2011). IL-1beta and IL-8 gave robust measurements for all 20 patients. Measurements were possible for between 10 and 15 patients for GM-CSF, IL-6, IFN-gamma and TNF-alpha. Other levels were below the limits of detection.

Cytokines were found to be raised 4 hours after force application (Table 3 and Supplemental Figure 2 A and B). Changes reached significance over baseline levels at tension sites for IL-1beta, IL-8 and TNFalpha and at compression sites for IL-1beta and IL-8. Levels of these cytokines at sites of tension remained significantly elevated after 7 and 42 days for IL-1beta and IL-8 and after 42 days for TNF-alpha, consistent with a prolonged inflammatory response. For the control second molars, GM-CSF and TNF-alpha were significantly increased 4-hours after force application, but at no other time points. There were positive correlations at sites of compression for IL-1beta ( $r=0.52$   $p=0.02$ ), IL-8 ( $r=0.55$   $P=0.01$ ) and TNF-alpha ( $r=0.78$   $P=0.001$ ) after placement of arch-wires but before force was applied, consistent with a contribution of plaque-induced inflammation to elevations in cytokines at this stage. No correlations were found with bleeding index scores. Positive correlations were found between IFNgamma and plaque index at sites of compression for second molars after placement of arch-wires, both before ( $r=0.73$   $p=0.009$ ) and 4 hours after force application ( $r=0.60$   $P=0.046$ ).

Cytokine measurements showed that IL-1beta quantities were significantly greater at canines after placement of arch-wires but before distalising force had been applied ( $P<0.05$ ), at 4 hours ( $p<0.01$ ); 7 days ( $P<0.01$ ) and 42 days ( $P<0.05$ ) after force application at tension sites and after 42 days only ( $P<0.05$ ) at compression sites.

IL-6 and IFN-gamma quantities at tension sites were significantly greater ( $P<0.05$ ) at the canines 4 hours after force application. GM-CSF was significantly greater at the canines after placement of arch-wires but prior to force application ( $P<0.05$ ) and 4 hours ( $P<0.01$ ) after a force was applied at compression sites.

TNF-alpha quantities were significantly greater at canine compression sites after arch-wire placement, but before force application ( $P<0.001$ ) and 4 hours ( $P<0.05$ ) after force application.



**Figure 2** Movement of teeth undergoing orthodontic treatment. A. Distance between canines and second molars. B. Speed of movement. Statistical analysis by Kruskal–Wallis: \*\*\* $P<0.001$ .



**Table 2** Clinical Values and scores for GCF volume, plaque and bleeding indices. Data shown as mean levels (pg/30s  $\pm$  the standard error of the mean).

Time	Canines				2nd molars			
	0	4 h	7 days	42 days	0	4 h	7 days	42 days
GCF Vol (ul)	0.72 $\pm$ 0.14	0.75 $\pm$ 0.08	0.71 $\pm$ 0.08	0.79 $\pm$ 0.09	1.01 $\pm$ 0.20	0.94 $\pm$ 0.14	1.04 $\pm$ 0.20	0.96 $\pm$ 0.16
tension								
GCF Vol (ul)	0.064 $\pm$ 0.10	0.64 $\pm$ 0.08	0.69 $\pm$ 0.08	0.64 $\pm$ 0.08	1.11 $\pm$ 0.17	1.21 $\pm$ 0.20	2.90 $\pm$ 0.17	0.72 $\pm$ 0.15
compression								
Plaque index	3.4 $\pm$ 0.6	2.4 $\pm$ 0.4	3.8 $\pm$ 0.6	3.3 $\pm$ 0.4	0.2 $\pm$ 0.2	0.2 $\pm$ 0.2	0.2 $\pm$ 0.2	0.6 $\pm$ 0.3
tension								
Plaque index	0.7 $\pm$ 0.2	1.2 $\pm$ 0.3	1.3 $\pm$ 0.3	1.3 $\pm$ 0.3	4.8 $\pm$ 0.8	2.3 $\pm$ 0.6	3.6 $\pm$ 0.8	3.8 $\pm$ 0.8
compression								
Bleeding index	1.3 $\pm$ 0.2	1.2 $\pm$ 0.2	0.6 $\pm$ 0.2	0.7 $\pm$ 0.2	0.2 $\pm$ 0.2	0.2 $\pm$ 0.2	0.2 $\pm$ 0.2	0.6 $\pm$ 0.3
tension								
Bleeding index	0.3 $\pm$ 0.1	0.5 $\pm$ 0.2	0.5 $\pm$ 0.1	0.4 $\pm$ 0.1	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0.1 $\pm$ 0.1
compression								

Significant positive correlations between cytokine levels and the speed of tooth movement were found at canines under tension for 4 hours for TNF-alpha ( $r=0.52$   $P=0.048$ ) and GM-CSF ( $r=0.57$   $P=0.03$ ). IL-1beta ( $r=0.435$   $P=0.055$ ) and IL-8 ( $r=0.376$   $P=0.102$ ) approached significance.

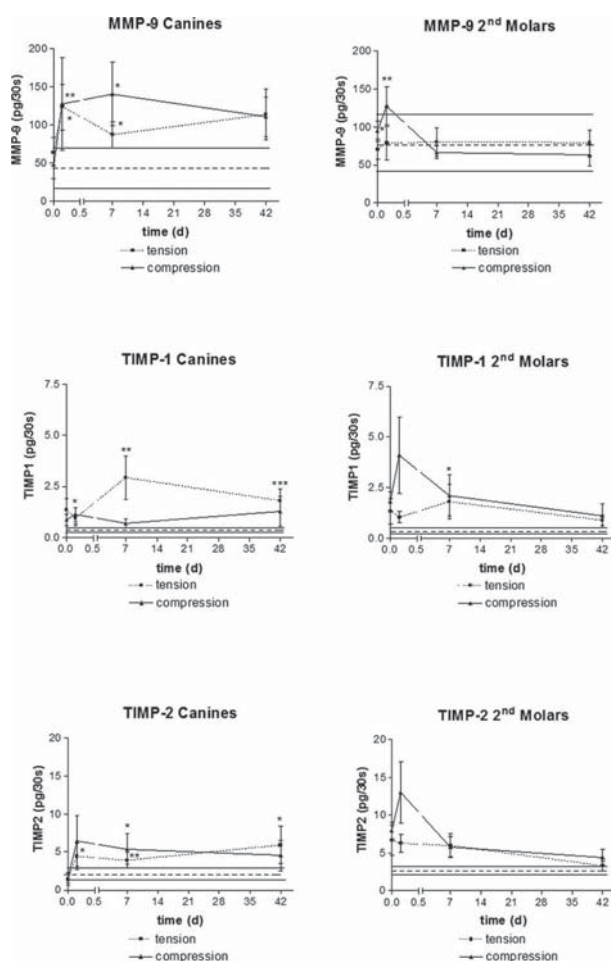
MMP-9, TIMP-1 and TIMP-2 proteins were measured to determine the level of tissue remodelling during orthodontic tooth movement. At canines, MMP-9 was significantly increased above baseline levels at sites of tension and compression 4 hours and 7 days after a distalising force was applied (Figure 3). TIMP 1 and 2 were significantly increased at sites of tension 4 hours, 7 days and 42 days after force application. TIMP-1 was significantly increased at sites of compression at 4 hours and TIMP-2 was significantly increased at sites of compression after force

application at 7 days. MMP-9 was significantly increased at the second molars under compression after placement of arch-wires but prior to force application and 4 hours after force application. TIMP-1 was found to be significantly increased 7 days after a force was applied at compression sites only. Ratios of MMP-9 to either TIMP-1 or 2 did not reach significance, however prolonged increases in the MMP-9/TIMP-1 ratio were seen at compression sites on the canines after force application (Supplemental Figure 3), consistent with a net tissue loss.

No correlations were found between plaque and bleeding indices for MMP-9 or TIMP-1 and 2 levels. Significantly more MMP-9 was found at canine compression sites than at second molars after placement of arch-wires before force application ( $p<0.01$ ). Significantly more TIMP-2 was found

**Table 3** Cytokine quantities for sites of tension and compression from canines and second molars. Data shown as mean levels (pg/30s  $\pm$  the standard error of the mean).

Time	Canines				2nd molars			
	0	4h	7 days	42 days	0	4h	7 days	42 days
IL-1b tension	0.05 $\pm$ 0.012	0.07 $\pm$ 0.011	0.06 $\pm$ 0.009	0.07 $\pm$ 0.011	0.02 $\pm$ 0.009	0.03 $\pm$ 0.014	0.02 $\pm$ 0.012	0.03 $\pm$ 0.020
IL-1b	0.04 $\pm$ 0.007	0.06 $\pm$ 0.010	0.05 $\pm$ 0.009	0.05 $\pm$ 0.009	0.02 $\pm$ 0.010	0.03 $\pm$ 0.010	0.02 $\pm$ 0.015	0.02 $\pm$ 0.010
compression								
GM-CSF	0.002 $\pm$ 0.001	0.006 $\pm$ 0.003	0.003 $\pm$ 0.001	0.005 $\pm$ 0.002	0.012 $\pm$ 0.002	0.016 $\pm$ 0.005	0.007 $\pm$ 0.002	0.005 $\pm$ 0.001
tension								
GM-CSF	0.001 $\pm$ 0.001	0.006 $\pm$ 0.004	0.005 $\pm$ 0.004	0.004 $\pm$ 0.001	0.012 $\pm$ 0.002	0.021 $\pm$ 0.004	0.009 $\pm$ 0.004	0.013 $\pm$ 0.006
compression								
IL-8 tension	0.151 $\pm$ 0.026	0.22 $\pm$ 0.027	0.20 $\pm$ 0.022	0.19 $\pm$ 0.030	0.298 $\pm$ 0.047	0.207 $\pm$ 0.033	0.166 $\pm$ 0.038	0.155 $\pm$ 0.038
IL-8	0.18 $\pm$ 0.028	0.18 $\pm$ 0.023	0.16 $\pm$ 0.024	0.15 $\pm$ 0.030	0.283 $\pm$ 0.046	0.325 $\pm$ 0.059	0.206 $\pm$ 0.051	0.187 $\pm$ 0.060
compression								
TNFa tension	0.032 $\pm$ 0.008	0.082 $\pm$ 0.023	0.075 $\pm$ 0.025	0.106 $\pm$ 0.040	0.049 $\pm$ 0.012	0.104 $\pm$ 0.046	0.027 $\pm$ 0.008	0.028 $\pm$ 0.007
TNFa	0.019 $\pm$ 0.005	0.096 $\pm$ 0.060	0.070 $\pm$ 0.038	0.079 $\pm$ 0.032	0.128 $\pm$ 0.028	0.180 $\pm$ 0.043	0.069 $\pm$ 0.025	0.137 $\pm$ 0.050
compression								
IL-6 tension	3.31 $\pm$ 0.13	38.3 $\pm$ 13.92	5.98 $\pm$ 1.27	16.2 $\pm$ 5.28	41.4 $\pm$ 36.5	45.6 $\pm$ 33.1	6.0 $\pm$ 1.7	15.7 $\pm$ 9.9
IL-6	4.48 $\pm$ 1.10	25.79 $\pm$ 13.91	9.71 $\pm$ 3.15	4.44 $\pm$ 0.86	10.6 $\pm$ 3.7	40.9 $\pm$ 9.0	55.3 $\pm$ 50.4	43.3 $\pm$ 29.1
compression								
IFNg tension	43.6 $\pm$ 15.2	89.8 $\pm$ 39.3	33.5 $\pm$ 12.2	51.5 $\pm$ 22.6	18.0 $\pm$ 8.9	26.9 $\pm$ 13.2	15.5 $\pm$ 7.1	24.7 $\pm$ 11.7
IFNg	42.1 $\pm$ 11.4	55.6 $\pm$ 28.9	65.2 $\pm$ 32.9	42.5 $\pm$ 14.7	26.5 $\pm$ 12.6	60.0 $\pm$ 28.2	19.5 $\pm$ 8.4	34.2 $\pm$ 17.6
compression								

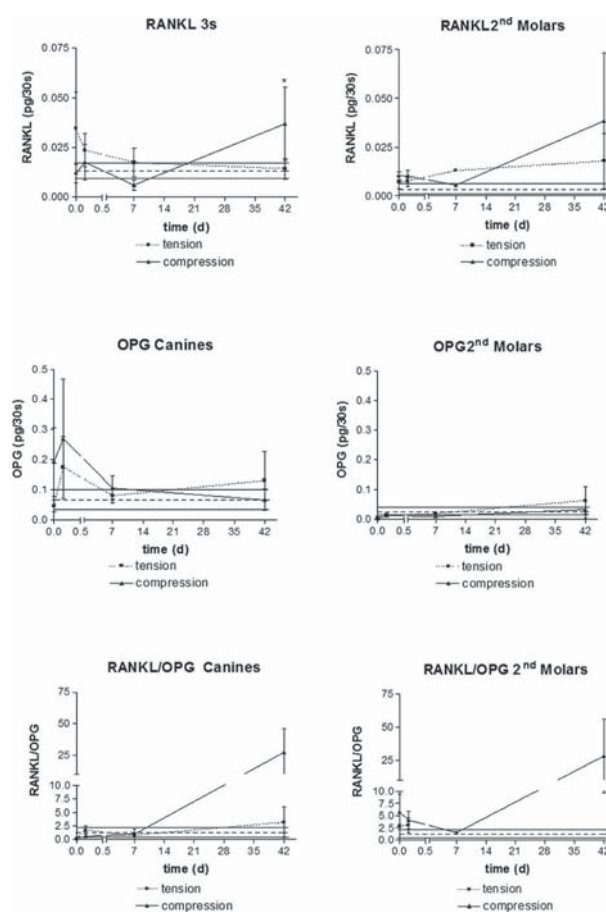


**Figure 3** Tissue metabolism biomarker quantities for sites of tension (dotted) and compression (solid) from canines and 2nd molars. Dashed lines represent mean values at baseline and parallel solid lines represent the SEM of the baseline values. Statistical analysis by Kruskal-Wallis: \* $P<0.05$ ; \*\* $P<0.01$ ; \*\*\* $P<0.001$ .

at sites of tension and compression at canines compared to second molars after placement of arch-wires before force had been applied (both  $p<0.01$ ).

When correlating MMP-9 and TIMP levels to speed of tooth movement, significant positive correlations were found at canines under tension 4 hours after force application for MMP-9 ( $r=0.58$   $p=0.008$ ) and TIMP-2 ( $r=0.7$   $p=0.0008$ ). TIMP-1 approached statistical significance ( $r=0.43$   $p=0.07$ ).

RANKL and OPG proteins were used to measure the level of bone remodelling during orthodontic tooth movement. RANKL was significantly increased at canine sites of compression 42 days after force application (Figure 4). There was a trend for RANKL also to be increased after 42 days at compression sites on second molars. OPG showed non-significant increases 4 hours after force application at canine sites of both tension and compression. No significant correlations were found for these biomarkers with the speed



**Figure 4** Bone metabolism biomarker quantities and ratios for sites of tension (dotted) and compression (solid) from canines and 2nd molars. Dashed lines represent mean values at baseline and parallel solid lines represent the SEM of the baseline values. Statistical analysis by Kruskal-Wallis: \* $P<0.05$ ; \*\* $P<0.01$ ; \*\*\* $P<0.001$ .

of tooth movement. However, at sites of compression the ratio of RANKL to OPG increased dramatically in the limited number of samples where both analytes were measured, reflecting a rise in RANKL and indicating increased bone catabolism.

## Discussion

Simultaneous changes were found in the quantities of cytokines and biomarkers of tissue and bone metabolism within GCF from teeth undergoing orthodontic tooth movement. Detailed clinical indices were recorded to account for confounding issues, such as increased plaque accumulation following appliance placement and resultant periodontal inflammation. Significant increases in GCF volume were found at canines under tension and compression forces, as has been previously reported (Samuels *et al.*, 1993; Baldwin *et al.*, 1999). It is thought that there is an immediate effect on blood vessels, giving rise to an increase in GCF volume via the serum transudate (Baldwin

*et al.*, 1999). Few studies have recorded periodontal indices but those that have, report little change (Yamaguchi *et al.*, 2006; Giannopoulou *et al.*, 2006). In the present study, bleeding was significantly increased only at placement of distalising force, demonstrating an immediate effect on the soft tissues. The significant correlation between plaque index and IL-1beta, IL-8 and TNF-alpha at distal canine sites post-arch-wire placement but pre-orthodontic force application implies a contribution of plaque-induced inflammation to cytokine levels

The greatest speed of tooth movement was seen 4 hours after the application of distalising force. Movement then slowed over 7 and 42 days, perhaps illustrating that at 4 hours the tooth was in the first phase of movement and that the subsequent time points were during the second and third phases (Burstone, 1962). Cellular and tissue reactions start in the initial phase of tooth movement, immediately post-force application, because of the compression and stretch of fibres and cells in PDL. Hyalinised zones are seen in pressure areas during the second phase with a distorted appearance of the normal PDL fibre arrangement. Disruption in blood flow produces arrest of tooth movement, which can last from 4 to 20 days (Krishnan & Davidovitch, 2006). Movement resumes only after removal of necrotic tissue and bone resorption from adjacent marrow spaces.

The cytokines IL-1 beta, IL-8 and TNF alpha were increased at sites under tension and compression on the canines to which force was applied. Increases in TNFalpha, GM-CSF, IL-1beta and IL-8 were correlated to speed of movement, indicating a rapid response to force application. Whether this reaction is inflammatory or not is a subject for debate. Meikle (2006) suggested that tooth movement met only the last of the four classical criteria for inflammation (redness, heat, swelling and pain), suggesting instead that the process was best regarded as an exaggerated form of normal physiological turnover combined with tissue repair. Cytokines function to recruit cells of the immune system and to modulate new bone formation. Increases in cytokines at the anchoring control second molar teeth imply that force affects the whole mouth, though this change was more transient than that seen at the canines. The increase in cytokines at the canines agrees with other studies which have shown that IL-1 beta and TNF- alpha are elevated in GCF collected from patients during the early phases of orthodontic tooth movement (Grieve *et al.*, 1994; Lowney *et al.*, 1995; Uematsu *et al.*, 1996; Dudic *et al.*, 2006). Dudic *et al.* (2006) also found that the biomarker patterns, particularly of IL-1beta, were different at tension and compression sites, with generally higher values of cytokines at tension sites. It may be that increases in pro-inflammatory cytokines at sites under tension are produced by osteoclasts responding to mechanical stress (Dudic *et al.*, 2006).

MMP-9 is a gelatinase that catabolises the tissue extracellular matrix. Chemical inhibition of this molecule will reduce orthodontic tooth movement (Holliday *et al.*, 2003;

Bildt *et al.*, 2007). Its activity is balanced by the production of physiological inhibitors TIMP-1 and TIMP-2. We have found high levels of MMP-9 throughout the observation period. The tissue inhibitors TIMP-1 and TIMP-2 were also raised, as found by Bildt *et al.*, (2009) using zymographic methods and by Capelli *et al.*, (2011) who recorded protein levels. MMP-9, TIMP-2 and TIMP-1 also correlated with speed of movement 4 hours after force application, highlighting the immediate response of the tissues to changes in the environment.

RANKL causes maturation of osteoclast precursors into mature osteoclasts; a process inhibited by osteoblastic production of OPG, which serves as a decoy receptor for RANKL, thereby inhibiting osteoclast differentiation. We have found that bone metabolism biomarkers were not significantly correlated with speed of movement; however the greatest changes in OPG levels were seen within 4 hours of force application. RANKL increased 42 days after force application. This may indicate activation of osteoclasts and bone resorption at the start of phase 3 of tooth movement. Kanzaki *et al.* (2002) found that culture media from compressed PDL fibroblasts stimulated osteoclastogenesis in peripheral blood mononuclear cell cultures and that RANKL mRNA expression was unregulated in those cells. OPG expression in contrast remained unchanged, as we found in this study. The ratio of OPG to RANKL around the second molar compression site was similar to that at compression canine sites, suggesting that bony resorption was occurring at both, around the same time scale. Yamaguchi *et al.* (2006) reported associations between increases in RANKL expression in compressed PDL cells and root resorption. Compressed PDL cells, obtained from patients demonstrating severe external apical root resorption, produced large amounts of RANKL, small amounts of OPG and stimulated osteoclast formation. Tyrovolas *et al.* (2008) described the link between RANKL levels, osteoclast activation and the resultant root resorption, whereby patients with high RANKL levels may suffer severe root resorption. It is possible that targeting optimal RANKL expression could create tooth movement without causing resorption.

## Conclusion

We conclude that during orthodontic tooth movement:

1. Tension sites adjacent to canines showed significant increases in IL-1beta, IL-8, TNFalpha from 4 hours to 42 days after application of force.
2. Statistically significant increases were detected in MMP9 during orthodontic tooth (canines) movement at sites of compression. The levels remained high from 4 hours until 7 days after application of force. This was mirrored by TIMP-2 levels, whereas TIMP-1 was elevated only 7 days after application of force at the tension side of the canines.



3. RANKL was statistically significantly increased only 42 days after application of orthodontic force.

## Supplementary Material

Supplementary figures can be accessed at [European Journal of Orthodontics online](#).

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